

Original Article



Quantitative Determination of Apigenin, Catalpol, and Gallic Acid in Total Extracts From Different Parts of Plantago Species by High-Performance Liquid Chromatography

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Abstract

Background: *Plantago* species have been used in traditional medicine to treat many types of diseases. The detection of apigenin, catalpol, and gallic acid in *Plantago lanceolata* and *Plantago major* has been optimized using this protocol.

Methods: The analyses were optimized using the C8 column, acetonitrile, and orthophosphoric acid–water (1:1%) as mobile phase at a flow rate of 1 mL.min⁻¹, and a wavelength detector was observed at λ 204 nm.

Results: The limits of detection (LOD) and quantification (LOQ) of the method were “0.04 and 0.14 µg/mL”, “0.007 and 0.022 µg/mL”, as well as “0.02 and 0.073 µg/mL” for catalpol, apigenin, and gallic acid, respectively. The highest level of apigenin in the dry weight of plants (4.34, and 1.99 µg/mg) was obtained from the spike and aerial parts of *P. lanceolata* and *P. major* species. High levels of gallic acid extracted from aerial parts and leaves of both species were 12.85 and 10.11 µg/mg, respectively. The highest amount of catalpol (43.33 and 18.15 µg/mg DW) was obtained from the spike of both *Plantago* sp. The calibration curves were linear with a correlation coefficient ($r > 0.9991$, 0.9996, and 0.9978).

Conclusion: In sum, the most simple and sensitive method to measure compounds was developed using HPLC, which showed a great validity.

Keywords: Flavonoid, Iridoid glycoside, LOD, LOQ, Phenol

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Introduction

The biological activities of plant extracts and, in particular, plants containing iridoids have received considerable research attention recently (1,2). Iridoids are a large group of monoterpenoids (3) that generally play a defensive role in plants (4). They are mostly assigned to the Lamiales such as *Plantago*, *Gentianales*, and *Cornales* (5). The iridoid glycosides aucubin and catalpol are among the biologically active secondary metabolites in plant extracts that can be utilized as chemotaxonomic and analytical markers for determining the quality of various plant extracts (6). The herbal substance contains about 2%-3% iridoid glycosides with aucubin and catalpol as the main compounds. The content of iridoid in old leaves is extremely low and, therefore, it is only used for tracking; while iridoid in young leaves even reached to a maximum of 9%. A study has reported that aucubin is the major compound in older leaves of plants, while catalpol is the dominant constituent in young leaves (7).

Apigenin is a subclass of flavonoids with very low toxicity (8). The hot infusion collected from *Plantago lanceolata* leaves has been discovered to include several polyphenols, such as flavonoids. The main compounds have been determined to be luteolin, apigenin, and luteolin-7-O-glucoside. The phenolic compounds, including vanillic and gallic acids, have been also recognized as the most abundant compounds (9).

Several flavonoids have been isolated from *Plantago major*, such as apigenin, luteolin, baicalin, baicalein, plantagin, homoplantagin, hispidulin, nepetin, and scutellarein (10,11).

Plantago genus belongs to the *Plantaginaceae* family, which includes about 275 species (12). *P. lanceolata* and *P. major* are the most well-known *Plantago* species, which possess numerous pharmaceutical properties.

Previous studies have demonstrated that apigenin, catalpol, and gallic acid are present in *P. lanceolata* and *P. major*. Therefore, our study aimed to optimize the



detection of catalpol, apigenin, and gallic acid using high-performance liquid chromatography (HPLC) in both *Plantago* species. The novelty of our study lies in the fact that it measured three compounds of iridoid, flavonoid, and phenol groups in different parts of the plants with the same simple method, which can encompass previous reports.

Materials and Methods

HPLC Method Conditions

The HPLC system included the pump (Waters Corp., Milford, MA, USA). UV detector (WATER, model Breeze, USA) was applied at the wavelengths of 204, 210, and 256 nm, and analyzed by Breeze software. The analysis of apigenin, catalpol, and gallic acid was conducted through two kinds of columns, Thermo Hypersil-C18 column (150 mm × 4.6 mm, 5 mm) and Phenomenex-C8 column (150 mm × 4.6 mm, 5 mm) equipped with the same packed guard column. The mobile phase included acetonitrile and orthophosphoric acid or formic acid (different ratios v/v) with a 1 mL.min⁻¹ flow rate. Using a loop injector (Rheodyne 7725i, Cotati, CA, USA), the injection volume to the system (20 µL) was produced and the column temperature was kept at 25°C.

Materials

Three compounds, including apigenin (98%), catalpol (96%), and gallic acid (96% purity) were provided by Sigma-Aldrich (Chemie GmbH, USA).

Standard Curves

The amount of the working solutions for producing the curve points equivalent to 1.5625-100 µg/mL catalpol and 0.15625-10 µg/mL apigenin and gallic acid was determined. The standard curve was obtained by plotting the peak areas against the concentrations of analytes in working solutions.

Method Validation

The well-organized method was predetermined by following ICH and USP instructions for validating analytical methods (13). To this end, the procedure was checked for limits of detection (LOD) and quantification (LOQ), and linearity on the basis of FDA guidance for industry.

Linearity. The blank samples were analyzed to assure that no interferences existed, however this analysis was not utilized for calibration function. Standard curves of seven concentrations of standards were examined. The LOD and LOQ were calculated using the following equation:

$LOD = \frac{3\sigma}{S}$, $LOQ = \frac{10\sigma}{S}$ here σ is the standard deviation of response and S is the slope of a calibration curve.

Preparation of Extracts

Plantago lanceolata and *P. major* were collected from the University of Zanjan, Iran. The voucher specimens were

prepared in triplicate and authenticated at the Botany Department, University of Zanjan.

Extraction of Different Parts of *Plantago* sp.

Thirty grams of smoothly ground herbal samples leave, aerial parts (except for spike), spike, and root parts were extracted using 80% aqueous methanol (600 mL) in 24 hours. at a room temperature (14).

The extracts were concentrated using a rotary evaporator under a vacuum and dried at room temperature for one week. Dried extracts were diluted in HPLC grade methanol to obtain stock solutions, and each extract solution was then passed through the sterile filter paper (Whatman filter paper, 0.22 µm) and stored in an amber vial at 4°C.

Statistical analysis

The results were reported as mean values. The means were compared using the Duncan test (P value < 0.05) by statistical SPSS version 21.

Results

Plantago species have many bioactive constituents including flavonoids, phenolic acids, triterpenes, phenylpropanoids glycosides, and iridoids as the main bioactive compounds. Some of the iridoid glycosides are regarded as the chemotaxonomic markers in *Plantago* genus.

Out of these compounds, three compounds of various classes, including iridoid glycoside catalpol, flavonoid apigenin, and phenol gallic acid were selected for conducting the current study.

Optimization of HPLC Conditions

Chromatographic variables, including column type and UV wavelengths, and mobile phase compositions, were optimized for catalpol, apigenin, and gallic acid assay by HPLC-UV.

Method Validation

Two kinds of columns, Thermo Hypersil-C18 column (150mm×4.6 mm, 5 mm) and Phenomenex-C8 column (150 mm×4.6 mm, 5 mm) were used. Using these two columns resulted in good peak separation and a shorter time of analysis. Several UV wavelengths were selected at 204, 210, and 256 nm. There was no significant difference between 204 and 210 nm; however, the peaks attributed to 204 nm displayed sharper peaks. The optimum separation, including good peak separation and a shorter time of analysis, was obtained for the C8 column with a flow rate of 1.0 mL.min⁻¹.

Furthermore, other chromatographic variables, including the mobile phase, were optimized for apigenin, catalpol, and gallic acid assay. Various mobile phase compositions with different concentrations of acetonitrile, methanol, and formic acid or orthophosphoric acid as eluents in water were compared to achieve the most proper separation. The results indicated that using 1%

acetonitrile–1% orthophosphoric acid in water system yielded the best resolution and sharp peaks.

The results also showed that the analytes elution times varied by adding various concentrations of acetonitrile–orthophosphoric acid in the mobile phase system. When the concentration of acetonitrile was increased to 5 and 10%, the retention time (Rt) decreased, which may have interfered with the solvent peak. In this study, 1% of acetonitrile was used in the last mobile phase system to shorten the running time of detection without interference.

The HPLC chromatogram of *P. lanceolata* and *P. major* confirmed the presence of apigenin, catalpol, and gallic acid at the Rt of around 2.5, 3.7, and 5.7 min, respectively; it also determined the relative standard deviation values of the Rt of 1.88, 0.88, and 0.76% for them, respectively.

Despite the nature of the method used to assay apigenin, catalpol, and gallic acid, further attempts were made to validate an optimal method as follows:

Linearity, LOD, and LOQ. Linear responses were observed for apigenin, catalpol, and gallic acid in the given concentration range which was appropriate for designated objectives. Figure 1 presents the regression coefficient (R) for apigenin, catalpol, and gallic acid. X and y in the typical linear regression equation for apigenin, catalpol, and gallic acid show the concentration of compound ($\mu\text{g}/\text{mL}$) and the peak area, respectively. Table 1 displays LOD and LOQ for apigenin, catalpol, and gallic acid.

HPLC Analysis of the Crude Extractions

The results revealed that under the 204 nm UV, the extracts contained various compounds among which apigenin, catalpol, and gallic acid were represented. Additionally, the analyte showed no interferences per sample (Figure 2).

Determination of Apigenin, Catalpol, and Gallic Acid Content in Crude Extracts of *Plantago* sp. Different Parts

The frequent presence and content of flavonoid, phenol, and iridoid glycoside in methanolic extracts from different parts of two medicinal plants *Plantago* sp, including the medicinal plant *P. lanceolata* and *P. major*, were examined by adopting HPLC method.

Figure 3 and Table 2 display the chromatogram and

Table 1. Validation of Analytical Method, Linearity Ranges, Limits of Detection, and Quantification

Standard Chemical	Regression Coefficient (R^2)	Concentration ($\mu\text{g}/\text{mL}$)	LOD	LOQ
Apigenin	$R^2 = 0.9996$	0.15625	0.00729	0.02211
Catalpol	$R^2 = 0.9991$	1.5625	0.04949	0.14997
Gallic acid	$R^2 = 0.9978$	0.15625	0.024357	0.07381

LOD, limits of detection; LOQ, limits of quantification.

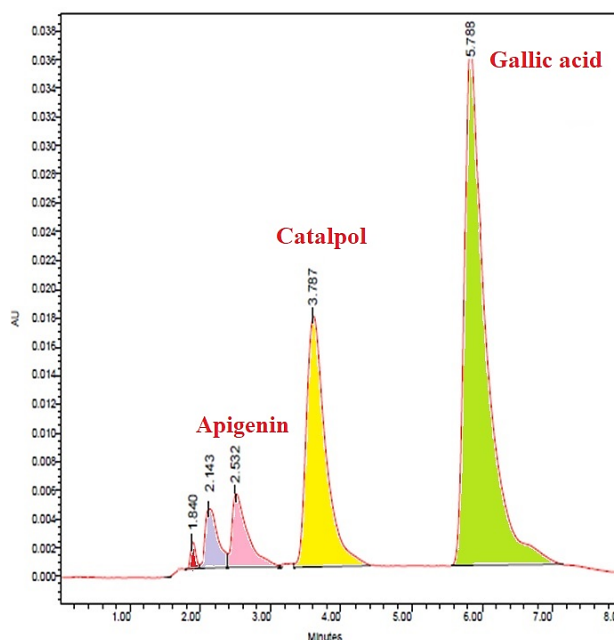


Figure 2. HPLC Chromatograms Developed for Apigenin, Catalpol, and Gallic Acid Assay Chromatogram of Standards With Concentrations of 1.25, 50, and 10 $\mu\text{g}/\text{mL}$ of Three Compounds. The two peaks are related to acetonitrile and methanol (1.84 and 2.14 min).

recovery (%) of crude extracts determined for apigenin, catalpol, and gallic acid extracted from two species of *Plantago* genus.

The highest level of apigenin [4.34, and 1.99 $\mu\text{g}/\text{mg}$ dry weight (DW) of plants] was obtained from spike and aerial parts of *P. lanceolata* and *P. major* species (Table 2). The amount of gallic acid was higher in both species in comparison to that of apigenin. High levels of gallic acid were obtained from the aerial part of *P. lanceolata* and

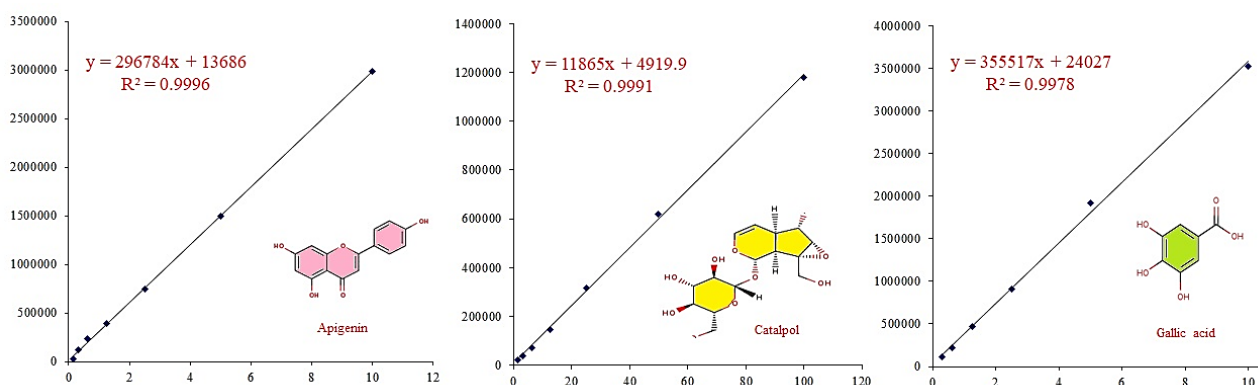


Figure 1. Calibration Curves, the Regression Coefficient of Apigenin, Catalpol, and Gallic Acid.

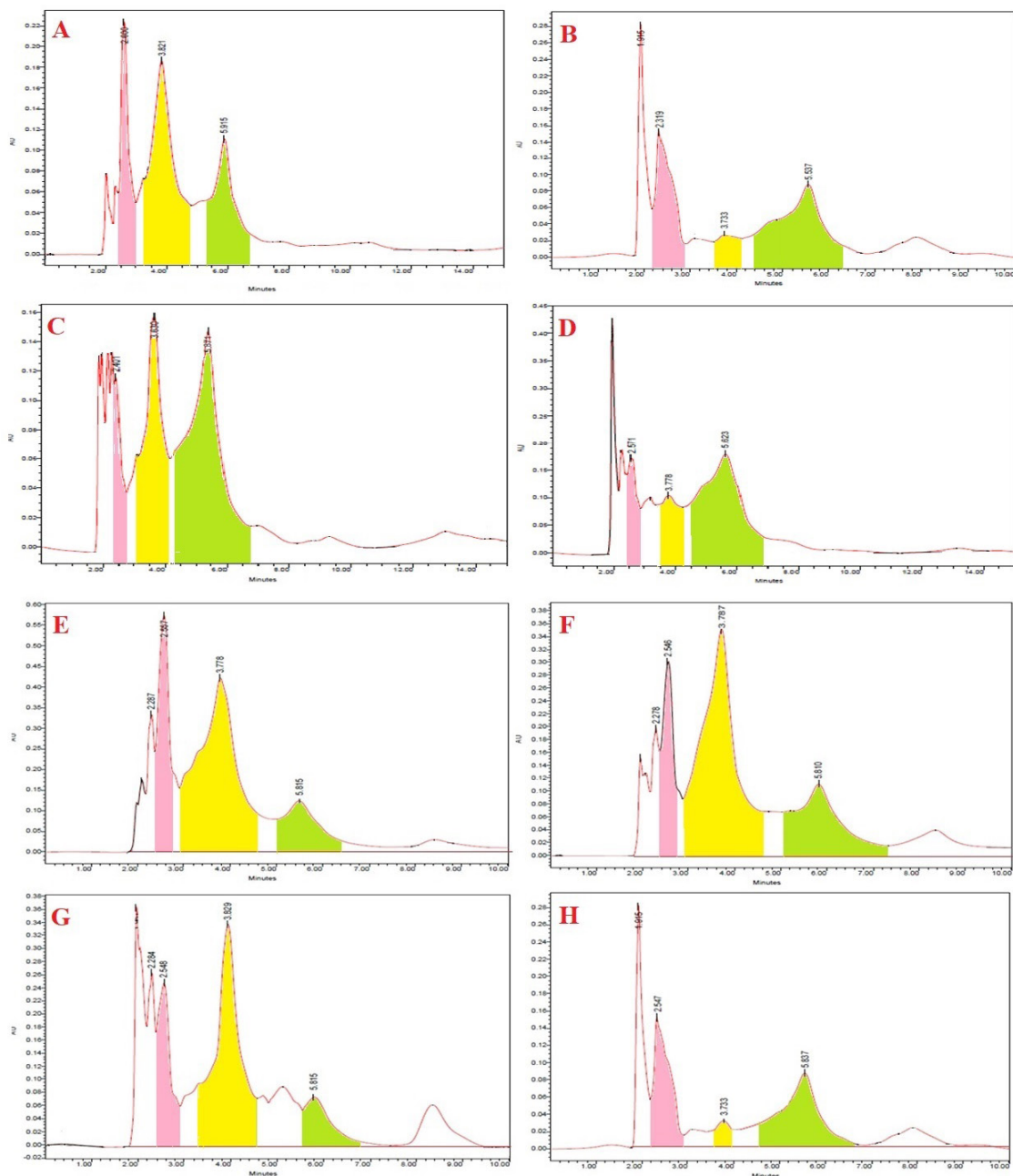


Figure 3. HPLC Chromatograms of Apigenin, Catalpol and Gallic Acid in Different Parts of *Plantago lanceolata* and *Plantago major*: (A and B) Chromatogram of methanolic extract from aerial part of *P. lanceolata* and *P. major*, (C and D) Methanolic extract from root part of *P. lanceolata* and *P. major*, (E and F) Methanolic extract from spike part of *P. lanceolata* and *P. major* (G and H) Methanolic extract from leaf part of *P. lanceolata* and *P. major*.

Table 2. The Extraction Recovery Determined for Apigenin, Catalpol, and Gallic Acid From Different Parts of *Plantago* sp

Different Parts	Apigenin (1) (µg/ mg ⁻¹ DW)	Apigenin (2) (µg. mg ⁻¹ DW)	Catalpol (1) (µg.mg ⁻¹ DW)	Catalpol (2) (µg.mg ⁻¹ DW)	Gallic Acid (1) (µg. mg ⁻¹ DW)	Gallic Acid (2) (µg. mg ⁻¹ DW)
Aerial	3.8146 ^b	1.0554 ^c	36.607 ^b	4.17312 ^c	12.8517 ^a	3.7002 ^c
RSD (%)	0.2098	0.3800	0.0331	0.5964	0.1990	0.2786
Spike	4.3430 ^a	1.2524 ^b	43.3382 ^a	18.15918 ^a	6.5841 ^b	3.8433 ^b
RSD (%)	0.0500	0.2509	0.0664	0.2686	0.3497	0.0462
Leaf	2.3327 ^c	1.9944 ^a	31.8837 ^c	1.73401 ^d	6.0322 ^c	10.1130 ^a
RSD (%)	0.2602	0.0655	0.0024	0.1748	0.3435	0.8238
Root	1.6853 ^d	0.5218 ^d	24.1659 ^d	10.91792 ^b	3.0097 ^d	3.1567 ^d
RSD (%)	0.0646	0.3256	0.1611	0.0084	0.3064	0.0384

RSD, relative standard deviation.
Mean of values ± standard deviation (SD) (n=3). (1) *Plantago lanceolata*; (2) *Plantago major*
Values followed by the same letter in a column are not significantly different.

P. major leaf. The amounts of gallic acid extracted from aerial parts and leaves of both species were 12.85 and 10.11 µg/mg DW, respectively. The highest amount of catalpol [43.33 and 18.15 µg/mg DW] was obtained from the spike of both *Plantago* sp.

The study results were summarized as follows: the amounts of apigenin, gallic acid, and catalpol in the aerial parts were higher than those in the root part of *P. lanceolata*. The contents of apigenin and gallic acid in the aerial parts and the content of catalpol in the root part of *P. major* were found to be higher. Generally, the yield of catalpol was higher than those of apigenin and gallic acid in both species of *Plantago*.

The yield of secondary metabolites (e.g., flavonoid apigenin, iridoid glycoside catalpol, and phenol gallic acid) in the plant species varies depending on many factors, including a pattern of climatic temperature during the plant growing season, harvest time, drying method, collection area, and environmental stresses.

Discussion

There are species of flowering plants in the family of *Plantaginaceae*, among which *P. lanceolata* and *P. major* are the most well-known species and have been widely distributed around the world for centuries. These species of the *Plantago* genus have been recognized as medicinal plants in Iranian traditional and modern medicine, and have been the focus of research attention in numerous studies. In the present study, three medicinal compounds of apigenin, catalpol, and gallic acid in methanolic extracts from different parts of *Plantago* species were evaluated by adopting a specific and reliable HPLC technique. In sum, a sensitive HPLC method was validated by the C8 column, a flow rate of 1 mL.min⁻¹, and the mobile phase consisted of acetonitrile: orthophosphoric in water acid (1:1% v/v). Iridoid glycoside aucubin and catalpol are very common in *Plantago* sp. Methanol-water had been employed as the mobile phase in earlier studies in order to determine iridoid glycoside aucubin in some *Plantago* sp by HPLC (15,16); since methanol may have interfered with the detection, however, Wu et al had used acetonitrile instead of this compound (17). In our study, the methanol peak interfered with the standard peak when methanol was used as a mobile phase, while no peak was observed after applying acetonitrile as a mobile phase except the internal standard peak.

Several studies have assessed the three compounds of two *Plantago* sp by HPLC and have found that after injecting the sample, the peaks for catalpol are around 4.35 minutes (18), 5 minutes (19), and 13.9 minutes (20); while those for gallic acid are nearly 4.40 minutes (21) and 4.6 minutes in the previous HPLC assay of *P. lanceolata* (22). In our study and under optimum conditions, the shortest and longest Rt analyses were recorded for apigenin (2.5 minutes) and gallic acid (5.7 minutes), respectively.

The presence and the amount of flavonoid, phenolic, and iridoid glycoside compounds in methanolic extracts

from different parts of *P. lanceolata* and *P. major* were examined by adopting a reliable HPLC method. According to the results from the statistical analysis of crude extracts by HPLC, the levels of apigenin and gallic acid were higher in the aerial parts compared to those in the underground parts of both species. However, the root part of the *P. major* exhibited more catalpol content than the aerial parts and leaves, which was in contrast to the result found for the *P. lanceolata* species.

Conclusion

This protocol had some advantages, including a/an quick, easy, validated, and economic extraction project, as well as a short chromatographic run time. The novelty of the current study lies in the fact that it was the first to evaluate apigenin, catalpol, and gallic acid contents of two species of this genus in different parts by using a single method.

Comparing the apigenin, catalpol, and gallic acid contents in *P. lanceolata* and *P. major* with those in other medicinal plants revealed that the two species may have had potential, medicinal properties. It was recommended that further studies on biological activity should be accompanied by more detailed examinations of their chemical composition. Such studies were in progress during the studies period. Taking into account the wide distribution of *Plantago* sp. in Iran, it was also suggested that further studies should be conducted to investigate these species.

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Author's Contribution

SRH: Investigation, project administration, writing - original draft and formal analysis. KB and AS: Funding and supervision. NM: Editing the English version manuscript.

Conflict of Interests

Authors declare that they have no conflict of interests.

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